

BIODEGRADABLE POLY (ESTER URETHANE) UREA BIOMATERIALS FOR APPLICATIONS IN COMBAT CASUALTY CARE

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1. ABSTRACT

A family of biocompatible, biodegradable poly(ester urethane)urea (PEUUR) biomaterials has been developed that degrade to non-toxic by-products and support the attachment and proliferation of cells. These two-component materials are synthesized by reactive liquid molding, thus rendering them suitable for injection or casting into molds to form a desired shape. The clinical goal is to develop biologically active cast and injectable PEUUR biomaterials that promote bone wound healing and decrease the incidence of nonunions and infection.

2. RELEVANCE TO THE ARMY

Nonunions are a significant clinical problem for civilians and military personnel. A 2004 report (*Capturing the Full Power of Biomaterials for Military Medicine*)¹ published by the National Research Council recognizes the potential for further development of bioactive materials that promote bone healing and decrease the incidence of non-unions. Military applications of bone graft materials incorporating bioactives that promote bone healing will be practical and realistic over the next 3 – 5 years. The 2004 report lists desirable characteristics and properties for a material to promote bone healing:

- (1) *Enhancement of the development of new blood vessels* that serve to promote healing and preventing infections during bone and muscle repair.
- (2) *Ease of use* (e.g., pastes and injectables that harden and cure *in vivo*).
- (3) *Biodegradability*: degradation at a rate equal to that of bone healing *in vivo*.
- (4) *Bioactivity*: controlled release of a wound-healing accelerant over a period of 1 to 3 weeks.

Infections are a significant clinical problem for civilians and military. Early reports indicate that approximately 65% of the wounds from the Iraq conflict culture positive for bacteria.² Open fractures are a common source of these infections, and represent the most challenging to treat. In a previous conflict, 4 of 11 open

fractures became infected (3 of the 5 tibial fractures).³ The soldiers who had complications, such as infection or non-union, had the longest hospital stay.

3. MATERIALS AND METHODS

3.1 Materials

Methyl 2,6-diisocyanatohexane (lysine methyl ester diisocyanate, LDI) and lysine triisocyanate (LTI) were purchased from Kyowa Hakko USA (New York). Sulfated castor oil (Turkey red oil), calcium stearate, stannous octoate, glycerol, and ϵ -caprolactone were purchased from Aldrich (St. Louis, MO). Glycolide and DL-lactide were purchased from Polysciences (Warrington, PA). TEGOAMIN 33 tertiary amine catalyst was received from Goldschmidt (Hopewell, VA) and Coscat 83 bismuth catalyst was supplied by ChasChem, Inc. (Rutherford, NJ). Glycerol was dried at 10 mm Hg for 3 hours at 80°C prior to use⁴. ϵ -caprolactone was dried over anhydrous magnesium sulfate prior to use. All other materials were used as received.

MC3T3-E4 mouse embryonic calvarial osteoprogenitor cells were obtained from American Type Culture Collection (ATCC-Manassas, VA) and cultured in α -MEM purchased from Invitrogen (Carlsbad, CA). 10% fetal bovine serum and 1% penicillin/streptomycin were obtained from Invitrogen. Phosphate buffered saline (PBS) was purchased from Invitrogen. 100-mL spinner flasks, magnetic stirring bars (0.8 cm diameter x 4 cm long), and Multi Stir 4 magnetic stirrer were purchased from Bellco Glass (Vineland, NJ). Silicon tubing (No. 13) and silicone stoppers (No. 13) were purchased from Cole Parmer (Niles, IL). Kirschner wire (Trochar shaped tip on both ends, 0.9 mm diameter, 19.1 cm long) was purchased from surgicaltools.com (Mystic, CT). Trypan blue solution and FITC labeled phalloidin were purchased from Sigma-Aldrich. Live/Dead[®] Viability/Cytotoxicity Kit and CyQUANT[®] Cell Proliferation Assay Kit were purchased from Molecular Probes, Inc. (Eugene, OR). MEDPOR[®] (Porex Surgical

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Inc., Newnan, GA) medical-grade polyethylene, Interpore™ 200 hydroxyapatite (Irvine, CA), and tissue culture polystyrene were used as positive controls in the cell culture experiments.

3.2 Synthesis of PEUR cast resins and foams

Two-component polyurethanes were mixed using a Hauschild SpeedMixer™ DAC 150 FVZ-K (FlackTek Inc., Landrum, SC). Elastomeric porous foams and hard cast materials were prepared by reactive liquid molding of two components: (a) a lysine polyisocyanate resin, and (b) a hardener comprising a poly(ϵ -caprolactone-*co*-glycolide-*co*-lactide) triol.⁵ Foams were synthesized from a 900-Da triol. Additionally, water (the blowing agent), a sulfated castor oil stabilizer, a tertiary amine catalyst (TEGOAMIN 33), and a calcium stearate pore opener were added to the hardener component to control the pore size and morphology. For cast PEUR resins, the resin comprised a quasi-prepolymer synthesized from the lysine polyisocyanate and polyester triol. The hardener component consisted of a 300-Da triol and a bismuth catalyst (Coscat 83). The two components were mixed at 3300 rpm for 15 s and the resulting reactive liquid mixture cast into a mold where it was allowed to cure *in situ* for 18 h. Foams were cured at 37°C while cast resins were cured at 60°C.

3.3 PEUR degradation

Degradation experiments were performed by incubating triplicate 10-mg samples of PEUR foam in PBS at 37°C for specified periods of time up to 12 weeks. At a given time point, the sample was removed from the PBS, dried under vacuum for 48 h at 37°C, and weighed to determine weight loss as a function of time.

3.4 Cell culture assays

Cell culture studies were carried out using standard aseptic tissue culture techniques. MC3T3-E4 osteoblast cells were seeded on the PEUR foams dynamically in a spinner culture flask. Static culture was performed on cast resins and the rate of cell proliferation measured using CYQUANT® assay after days 1, 4, and 7.

The Live/Dead Viability/Cytotoxicity Kit (Molecular Probes, Invitrogen, Carlsbad, CA, L-3224) was used to determine the viability of attached cells on the cast and foam surface after 24 and 48 hours. The polymer degradation products were tested for cell viability and cytotoxicity with MC3T3 osteoblast-like cells for 24 and 48 hours. Briefly, 5×10^4 cells/well were seeded in a 24 well tissue culture plate containing 900 μ l α -MEM and 100 μ l polymer degradation products supplemented with 10 % fetal bovine serum, 1% penicillin (100U/ml), and streptomycin (100 μ g/ml). Controls were treated with 900

μ l α -MEM and 100 μ l PBS. Cytotoxicity was measured by Live/Dead staining and a commercially available LDH assay kit (CytoTox 96 Non-Radioactive Cytotoxicity Assay Protocol, Promega G1780), which measures lactate dehydrogenase (LDH), a stable cytosolic enzyme released upon cell lysis.

To evaluate *in vitro* attachment of cells to cast PEUR resins, MC3T3-E1 cells were seeded on the cast polymers for 48hrs and either fixed with 2.5% glutaraldehyde for SEM or 4% Paraformaldehyde for immunodetection of actin and Matrix Metalloproteinase-1 (MMP-1). Cells were stained with Phalloidin TRITC to visualize actin organization and cell anchorage to the cast polymer surface and counter stained for nucleus with hoescht blue.

For the osteoblast mineralization study, 5×10^4 MC3T3 osteoblast cells were seeded on the surface of the polymers and treated with osteogenic media (10mM β -glycerophosphate and 50mg/mL ascorbic acid) and assayed for osteoblast mineralization after 21 and 28 days. Tetracycline labeling (8 μ g/mL) and Alizarin red staining was performed to detect mineralization.

3.5 SEM-EDAX

Scanning Electron Microscopy - Energy Disperse X-ray Analysis was performed to detect biomineralization after 28 days of culture in osteogenic medium.

3.6 MicroFT-IR

The composition of the materials and biomineralization was assessed by micro FT-IR (Nicollet Coninuum™). After 28 days of cell culture on the foams the sample with the cells was fixed in 2.5 % glutaraldehyde and directly analyzed under the FT-IR microscope. The spectra was collected in the reflectance mode with a 4 cm^{-1} resolution and 150 scans.

4. RESULTS AND DISCUSSION

4.1 Summary of mechanical and physical properties

The elastic modulus of the glassy cast materials varied between 1200 – 1430 MPa and the compressive strength varied from 82 – 111 MPa.⁶ Elastomeric foam scaffolds cast as reactive liquid mixtures rose and gelled in approximately 5 - 15 minutes. By varying the concentration of water in the hardener, porous scaffolds with porosities ranging from 89 – 95% and pore sizes ranging from 200 – 1000 μ m were prepared.^{5, 7} The foams were soft with compressive stresses at 50% deflection ranging from 1 – 3 kPa. Elastomeric foams and glassy cast materials exhibited 0.5 – 12% mass loss after

12 weeks incubation in PBS at 37°C. The half-lives of the poly(ϵ -caprolactone-*co*-glycolide-*co*-lactide) triols incorporated in the PEUR materials controlled the degradation rate.

4.2 Attachment and viability

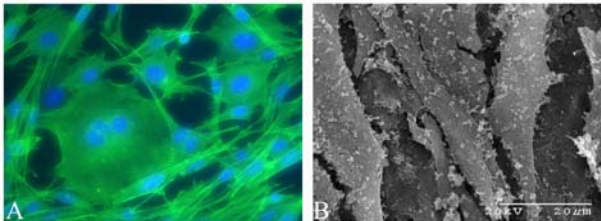


Fig 1A. Actin organization of MC3T3 cells seeded on the cast polymer labeled with phalloidin TRITC and nucleus stained with Hoechst blue. 1B. SEM image of MC3T3 cells.

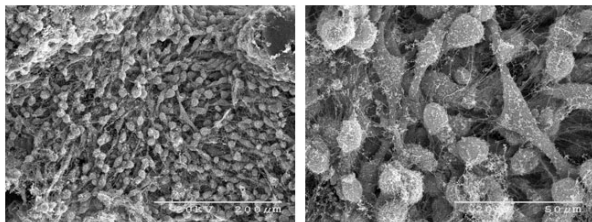


Fig 2. SEM images of MC3T3 cells seeded on PEUR foams.

MC3T3 cells attached to both cast and foam PEUR surfaces, as shown in Figures 1 and 2. Actin staining (Figure 1A) and SEM images (Figure 1B) showed comparable cell attachment to the cast polymers relative to the polystyrene control. Similarly, SEM images also revealed good cell attachment and cell ingrowth into the elastomeric foams, as shown in Figure 2. Cell proliferation assay showed increase in cell count from Day 1 to Days 4 and 7, implying that the MC3T3 cells

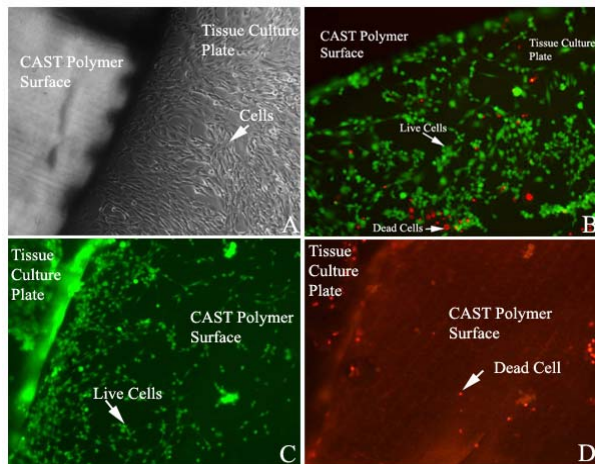


Figure 3. (A) MC3T3 cells closely apposed to the cast PEUR surface; (B) live and dead cells adjacent to the cast polymer; (C) live cells adhered to the cast polymer surface and (D) very few dead cells on the cast polymer surface. All the images were captured under 10x magnification.

proliferated on both the cast and foam materials.

Direct-contact cytotoxicity assay revealed that the surfaces of the cast PEUR materials supported the attachment of viable MC3T3 cells, as shown in Figure 3. More than 80% of the attached cells were viable. The phase contrast image (Figure 3A) demonstrates that the cells are closely apposed to the material. Figures 3B and 3C show viable cells around the disc and on the disc.

4.3 Cytotoxicity of degradation products

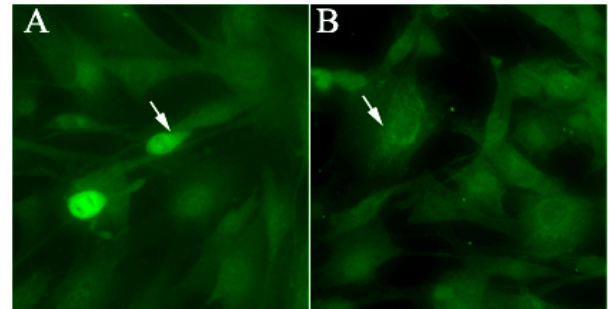


Figure 4. MMP-1 expression in MC3T3 Osteoblast Cells: (A) Control cells untreated and (B) Cells treated with polymer degradation product after 24hrs. Images were captured under 20x magnification.

LDH assay and live/dead staining of the cells after incubation with polymer degradation products showed 70 to 80 % viable cells after 24 and 48 hours (comparable to

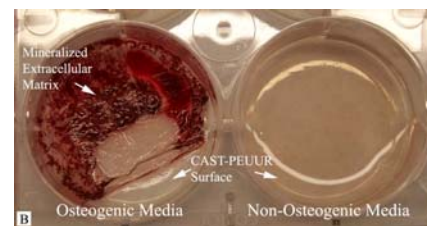
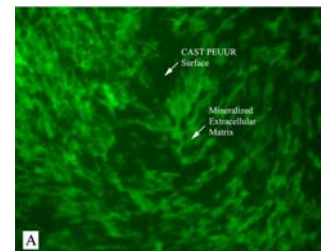


Figure 5. Tetracycline labeling (A) and Alizarin red staining (B) of the extracellular matrix after 28 days.

the control values), suggesting that the PEUR degradation products are non-cytotoxic. As shown in Figure 4, immunofluorescence staining of MC3T3 cells treated with polymer degradation products exhibited expression of MMP-1 comparable to that of untreated control cells.

4.4 Mineralization of cast PEUR surfaces

Tetracycline labeling and Alizarin red staining showed mineralized extracellular matrix after 21 and 28 days on CAST PEUR surface treated with osteogenic media, whereas the cells cultured without osteogenic media did not show any ECM mineralization (Figure 5).

SEM-EDAX analysis of the PEUR foams revealed mineralized extracellular matrix and osteoblast cells (Figure 6) after 28 days of culture in osteogenic medium, suggesting that osteoblast differentiation and matrix mineralization was not impeded by the PEUR foams. Osteoblast mineralization was observed on the surface as well as deep inside the polymer, which implies the porous property of the material facilitated cell infiltration as well as mineralization. Table 1 show the presence of calcium and phosphorus in the mineralized matrix.

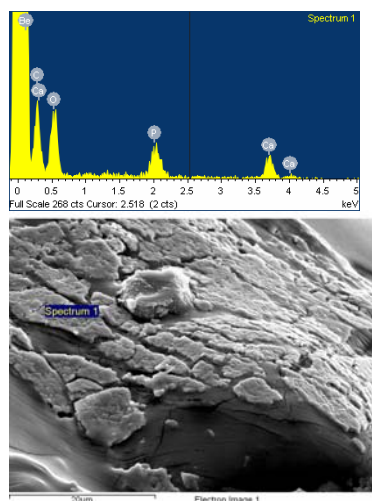


Figure 6. SEM and EDAX spectra of the mineralized matrix on the PEUR foam seeded with cells after 28 days culture in osteogenic media.

Element	Weight%	Atomic%
C K	28.79	40.52
O K	44.66	47.19
P K	8.76	4.78
Ca K	17.79	7.50
Totals	100.00	

Table 1. Calcium (CaK) and phosphorous (PK) are present in the mineralized matrix after 28 days on the PEUR foams

Micro FT-IR spectrum of the PEUR foams revealed the presence type B carbonate (CO_3^{2-}) substitution peaks

between 1469 cm^{-1} - 1436 cm^{-1} . Phosphate peaks (PO_4^{3-}) were detected between 961 cm^{-1} - 1167 cm^{-1} confirming the mineralization. The FTIR data also suggest that the PEUR foams degrade due to hydrolysis of ester linkages in the polyester polyol, thereby yielding α -hydroxy acids and soluble urethane and urea fragments.

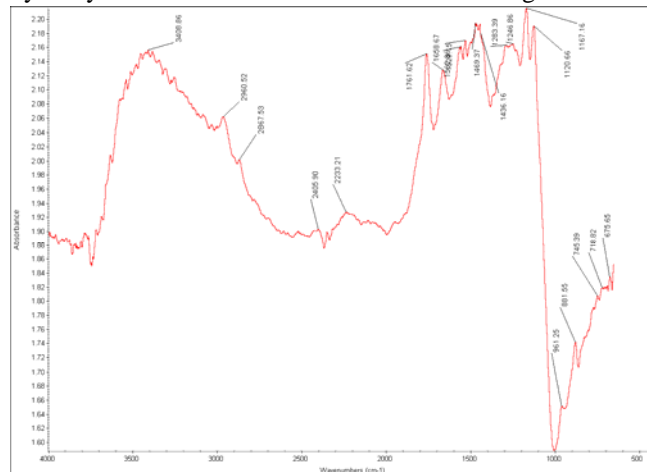


Figure 7. show microFT-IR of the mineralized matrix of PEUR foam seeded with cells after 28 days culture in osteogenic media

5. CONCLUSIONS

Biocompatible and biodegradable elastomeric foams and cast PEUR resins have been prepared from lysine-derived polyisocyanates and polyester triols by reactive liquid molding. The materials support the attachment and proliferation of MC3T3 cells and biodegrade to non-cytotoxic degradation products *in vitro*. These materials have potential application as injectable and castable delivery systems of biologically active molecules to enhance bone wound healing and control infection.

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